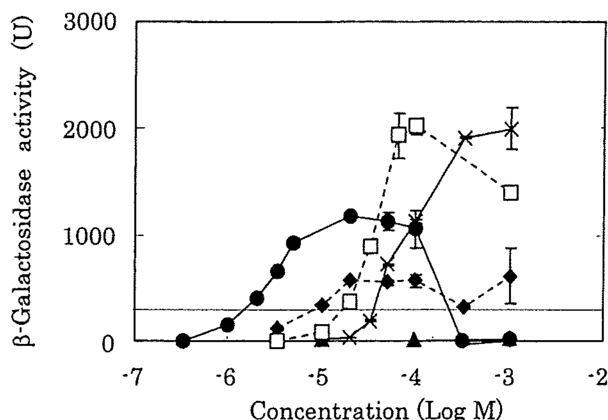

Fig. 4. Dose–Response Curves of Estrogenic Activity of Di- and Tri-Hydroxylated Benzophenones

Each point is the mean ± S.D. ($n = 3$). The dotted line shows 10% activity of 10^{-6} M 17β-estradiol. 2,2′-dihydroxybenzophenone (▲), 2,4-dihydroxybenzophenone (●), 4,4′-dihydroxybenzopenone (□), 2,3,4-trihydroxybenzophenone (◆), 2,4,4-trihydroxybenzophenone (×).

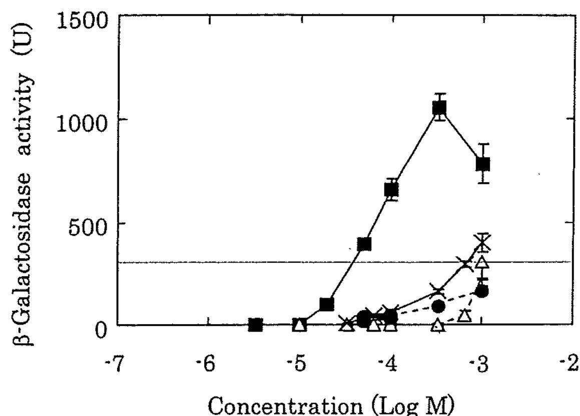

Fig. 6. Dose–Response Curves of Estrogenic Activity of Hydroxylated and Methoxylated Benzophenones

Each point is the mean ± S.D. ($n = 3$). The dotted line shows 10% activity of 10^{-6} M 17β-estradiol. 2-hydroxy-4-methoxybenzophenone (×), 2,2′-dihydroxy-4-methoxybenzophenone (△), 2,2′-dihydroxy-4,4′-dimethoxybenzophenone (●), 4-hydroxy-2′,4′-dimethoxybenzophenone (■).

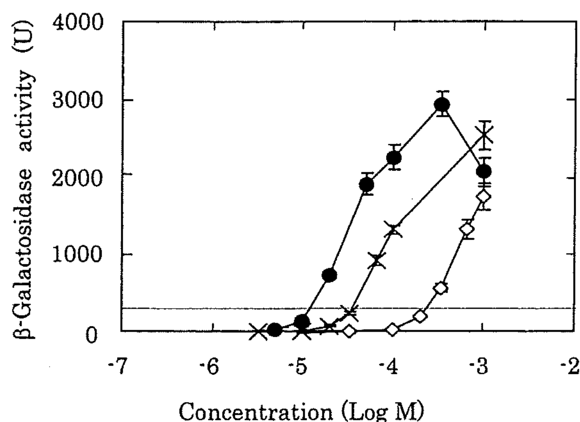

Fig. 5. Dose–Response Curves of Estrogenic Activity of Tetra-Hydroxylated Benzophenones

Each point is the mean ± S.D. ($n = 3$). The doted line shows 10% activity of 10^{-6} M 17β-estradiol. 2,2′,4,4′-tetrahydroxybenzophenone (●), 2,3,4,4′-tetrahydroxybenzophenone (×), 2,3′,4,4′-tetrahydroxybenzophenone (◇).

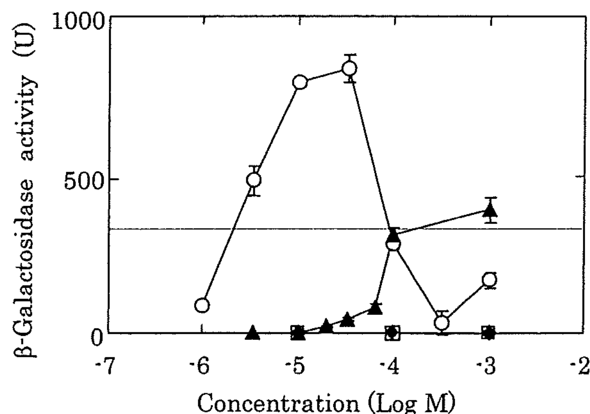

Fig. 7. Dose–Response Curves of Estrogenic Activity of the Other Hydroxylated Benzophenones

Each point is the mean ± S.D. ($n = 3$). The dotted line shows 10% activity of 10^{-6} M 17β-estradiol. 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid (□), 2-hydroxy-4-n-octyloxybenzophenone (◆), 2-hydroxy-5-methylbenzophenone (▲), 4-hydroxy-4′-chlorobenzophenone (O).

dimethoxybenzophenone (No. 16) was rather strong (4.0×10^{-5} M), but the others which did not possess a hydroxyl group at the 4-position had weak or negative activity (Fig. 6).

The activities of 2-hydroxy-4-methoxybeozophenone-5-sulfonic acid and 2-hydroxy-4-n-octyloxybenzophenone (No. 17, 18) were not determined, and 2-hydroxy-5-methylbenzophenone (No. 19) had weak activity. However, the activity of 4-hydroxy-4′-chlorobenzophenone (No. 20, 2.2×10^{-6} M) was as strong as that of 2,4-dihydroxybeozophenone (Fig. 7).

DISCUSSION

As shown in Table 3, 13 benzophenones were already reported to be estrogenic using other assay methods.[1,2,6,9–11] Comparing the present results to those, findings 8 relatively strong compounds for which REC_{10} values were under 10^{-4} M (No. 3, 4, 6–10, 20), and two negative compounds (No. 1, 5) were the same as in all the previous papers regarding positive or negative activity. Only three weakly positive compounds whose REC_{10} values were over

Table 3. Comparison of Estrogenic Activities of Benzophenones with Present and Previous Investigations

No.	Compound	Present investigation	Previous investigation						
		Yeast two-hybrid assay	MCF-7 cell assay		Competitive binding assay		Recombinant yeast assay[11]	Uterotropic assay	
		REC_{10} (M)	EC_{50}[1] (M)	positive[2,9] (M)	IC_{50}[2,10] (M)	IC_{50}[6] (M)	EC_{50} (M)	ED_{50}[1] (mg/kg/day)	positive[10] (mg/kg/day)
1	Benzophenone	$> 1.0 \times 10^{-3}$		$> 10^{-4}$	$> 5 \times 10^{-4}$		Nonactive		> 400
2	2-Hydroxy	6.2×10^{-4}					Nonactive		
3	3-Hydroxy	1.0×10^{-5}					2.57×10^{-6}		
4	4-Hydroxy	4.5×10^{-6}		10^{-5}	5×10^{-5}		1.12×10^{-6}		100
5	2,2′-Dihydroxy	$> 1.0 \times 10^{-3}$				$> 1.00 \times 10^{-4}$	Nonactive		
6	2,4-Dihydroxy	1.8×10^{-6}		10^{-8}	5×10^{-5}	3.65×10^{-5}			
7	4,4′-Dihydroxy	3.8×10^{-5}				2.60×10^{-5}	2.53×10^{-6}		
8	2,3,4-Trihydroxy	9.0×10^{-6}		10^{-7}	5×10^{-4}		5.08×10^{-6}		
9	2,4,4′-Trihydroxy	1.8×10^{-5}					5.64×10^{-7}		
10	2,2′,4,4′-Tetrahydroxy	1.4×10^{-5}					7.92×10^{-7}		
11	2,3,4,4′-Tetrahydroxy	3.6×10^{-5}							
12	2,3′,4,4′-Tetrahydroxy	2.4×10^{-4}							
13	2-Hydroxy-4-methoxy	6.6×10^{-4}	3.73×10^{-6}	$> 10^{-5}$	$> 1 \times 10^{-4}$	$> 1.00 \times 10^{-4}$		1000–1500	
14	2,2′-Hydroxy-4-methoxy	1.0×10^{-3}		10^{-7}	$> 1 \times 10^{-4}$	$> 1.00 \times 10^{-4}$			
15	2,2′-Hydroxy-4,4′-methoxy	$> 1.0 \times 10^{-3}$							
16	4-Hydroxy- 2′,4′-methoxy	4.0×10^{-5}							
17	2-Hydroxy-4-methoxy-5-sulfonic acid	$> 1.0 \times 10^{-3}$							
18	2-Hydroxy-4-*n*-octyloxy	$> 1.0 \times 10^{-3}$							
19	2-Hydroxy-5-methyl	1.3×10^{-4}							
20	4-Hydroxy-4′-chloro	2.2×10^{-6}					2.88×10^{-7}		

1, 2, 6, 9–11) : Refernce No.

10^{-4} M (No. 2, 13, 14), were found to be negative in some reports mainly by the competitive binding assay. These discrepancies seemed to be due to differences in sensitivity of methods. Thus, the present results were very consistent with previous results, though the strength of activity showed some variation.

The present study is the first report on the estrogenicities of 9 kinds of UV stabilizers and 7 kinds of hydroxylated benzophenones (No. 11, 12, 15–19). Among them, 4 kinds of benzophenones (No. 11, 12, 16, 19) were positive for estrogenic activity.

To predict estrogenic potency based on molecular structure, rules have been proposed and one was applied to benzophenone derivatives.[11] The predictions, however, did not fit our results well, especially regarding compounds hydroxylated only at the 2-position and symmetrical compounds.

A common structure for estrogenic compounds tested by the yeast two-hybrid assay was proposed by Nishihara[5] that most positive compounds have a phenol ring with a moiety of appropriate hydrophobicity at the *p*-position. The present results were a very good match with this rule. All of the 4-hydroxylated benzophenones (No. 4, 6–12, 16, 20), which had a phenol ring, and also had a benzoyl group as a hydrophobic moiety at the *p*-position, had rather strong activity. However, benzophenones without a hydroxyl group at the 4-position were negative (No. 1, 5, 15, 17, 18) or weakly positive (No. 2, 13, 14, 19). One exception was 3-hydroxybenzophenone which had no hydroxyl group at the 4-position, though its activity was rather strong. It was presumed that the two benzene rings of benzophenone were not fixed; therefore, the 3-hydroxyl group could bind the receptor to nearly the same degree as the 4-hydroxyl group. The hydroxyl group of the phenol moiety affects estrogenic activity in the order of the 4-position > 3-position >> 2-position of benzophenone.

Furthermore, when one more hydroxyl group at the 2-position was added to 4-hydroxylated benzophenones, the activity was enhanced such that No. 4 (REC_{10}: 4.5×10^{-6} M) < No. 6 (1.8×10^{-6} M), and No. 7 (3.8×10^{-5} M) < No. 9 (1.8×10^{-5} M). It was presumed that the 2-hydroxyl group interacted with the carbonyl group; the benzophenone structure then became fixed and the length of the hydrophobic moiety fitted the receptor. A hydroxyl group at the 3-position added to 4-hydroxylated benzophenones, however, might reduce the activity slightly such that No. 6 (1.8×10^{-6} M) > No. 8 (9.0×10^{-6} M) and No. 9 (1.8×10^{-5} M) > No. 11 (3.6×10^{-5} M).

On the other hand, when a hydroxyl group was added to the benzoyl moiety, the estrogenic activity was reduced drastically, with No. 2 (6.2×10^{-4} M) > No. 5 (nonactive), No. 4 (4.5×10^{-6} M) > No. 7 (3.8×10^{-5} M), No. 6 (1.8×10^{-6} M) > No. 9 (1.8×10^{-5} M) > No. 12 (2.4×10^{-4} M), No. 8 (9.0×10^{-6} M) > No. 11 (3.6×10^{-5} M), and No. 13 (6.6×10^{-4} M) > No. 14 (1.1×10^{-3} M). Commonly, estrogenic compounds possess two hydroxyl groups on opposite sides, *e.g.* estradiol, distylbesterol, bisphenol A, and both hydroxyl groups were presumed to contribute to the estrogenicity. However, our results indicated that the second hydroxyl group at the opposite ring would not only contribute to the estrogenicity but also reduce activity in the case of benzophenone derivatives.

One exception was No. 9 (1.8×10^{-5} M) < No. 10 (1.4×10^{-5} M), and it was presumed that the 2′-hydroxyl group interacted with the carbonyl group the same as the 2-hydroxyl group described previously, and the benzophenone structure might be fixed. While a chloro group added to the benzoyl moiety enhanced the activity, such that No. 4 (4.5×10^{-6} M) < No. 20 (2.2×10^{-6} M).

Regarding the methoxy group, the estrogenic activity was unchanged when it was added to phenol ring. While the activity might be reduced when the methoxy group was added to the benzoyl moiety, though examples were a few.

With these structure-activity relationships, the estrogenic activity of most of the benzophenones tested in the present study could be explained clearly. Moreover, it is presumed that some of these relationships have potential applications in other estrogenic chemicals which possess two benzene rings.

Fifteen hydroxylated benzophenones showed estrogenic activity, and four of them were more active than bisphenol A. The majority of these chemicals are used as UV stabilizers in plastics, sunscreens, cosmetics and others. They are also produced in the body as the metabolic products of benzophenone and its derivatives. Therefore, more investigations of hydroxylated benzophenones and their endocrine disrupting effects are needed.

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Estrogenicity of Metabolites of Benzophenone Derivatives Examined by a Yeast Two-Hybrid Assay

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The estrogenic activities of S-9 metabolites of benzophenone derivatives (benzophenone, 2-hydroxy-4-methoxybenzophenone, 2,2′-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-octyloxybenzophenone, 2,4-dihydroxybenzophenone and 2,3,4-trihydroxybenzophenone) and benzhydrol were examined with a yeast two-hybrid screening system. After chemicals were incubated in an S-9 mix at 37°C for 4 hr prior to their incubation with the yeast strain, the S-9 mix containing metabolites was assayed for the estrogenic activity by the yeast two-hybrid assay. Benzophenone, 2-hydroxy-4-methoxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone exhibited estrogenic activities after incubation with the S-9 mix. The estrogenic metabolites of 2-hydroxy-4-methoxybenzophenone were fractionated by high-performance liquid chromatography, one of which was identified as 2,4-dihydroxybenzophenone. This assay will be a useful tool for detecting proestrogens.

Key words —— yeast two-hybrid assay, benzophenone, estrogenic metabolite

INTRODUCTION

Benzophenone is listed among the "chemicals suspected of having endocrine disrupting effects" by the World Wildlife Fund, the National Institute of Environmental Health Sciences in the U.S.A., and the Ministry of Environment in Japan. However, benzophenone is an important compound in everyday life because of its ability to absorb and dissipate ultra violet (UV) light.[1] Its twelve derivatives, designated as benzophenone-1 through benzophenone-12, are used in cosmetics and sunscreens to protect human skin and hair from UV irradiation. 2-Hydroxy-4-methoxybenzophenone (benzophenone-3, BZ-3) is one of the most widely used UV absorbers for sunscreens on the market. Orally or topically administered BZ-3 is converted to at least three metabolites, 2,4-dihydroxybenzophenone (benzophenone-1, BZ-1), 2,3,4-trihydroxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone (benzophenone-8, BZ-8).[2–5] BZ-1 and 2,3,4-trihydroxybenzophenone exhibited estrogenic activities in an *in vitro* assay system using MCF-7 cells.[6] Benzophenone is converted to an estrogenic metabolite, *p*-hydroxybenzophenone.[7] Thus, benzophenone derivatives can be categorized as proestrogens. However, the estrogenic activities of the metabolites of benzophenone derivatives have not been fully elucidated.

We have developed a novel assay procedure for detecting the hormonal activities of chemicals using a yeast two-hybrid system.[8] We tested the estrogenic activities of various chemicals, and found that a phenol with a hydrophobic moiety at the para-position is the key structural moeity of estrogenic chemicals.[9] The phenyl or phenylether residues of lipophilic chemicals can be converted to a phenol residue by drug metabolizing enzymes. These facts imply that some chemicals exert their estrogenic activities by metabolic activation *in vivo*. The endocrine activities of pesticides and natural products can be affected by metabolism.[10,11] For example, methoxychlor (MXC) is metabolized to 2,2-*bis*(hydroxyphenyl)-1,1,1-trichloroethane to exert estrogenic activity.[12] The EDSTAC final report recommends that the evaluation of chemicals using *in*

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vitro high throughput prescreens should be performed in the presence and absence of metabolically active extracts to detect proestrogens.[13] There are few reports of assay procedures that are able to evaluate the estrogenic activity of metabolites.[14–16] In mutagenicity testing, incubation with an S-9 extract mixture has been the standard method for *in vitro* metabolic activation.[17] Here we apply a yeast two-hybrid assay for detection of estrogenic activity after metabolic activation by incubation with an S-9 extract mix (S-9 mix), and examine the estrogenic activities of metabolites of benzophenone derivatives.

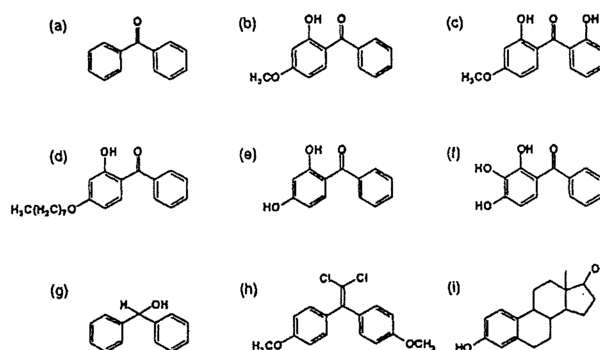

Fig. 1. The Structure of Chemicals Examined in This Assay
a), benzophenone; b), BZ-3; c), BZ-8; d), 2-hydroxy-4-octyloxybenzophenone (BZ-12); e), 2,4-dihydroxybenzophenone (BZ-1); f), 2,3,4-trihydroxybenzophenone; g), benzhydrol; h), MXC, i), E_2.

MATERIALS AND METHODS

Chemicals —— 17-β-Estradiol (E_2, > 97.0%), MXC (> 97.0%), benzophenone (> 98.0%), 2,4-dihydroxybenzophenone (> 98.0%), 2,3,4-trihydroxybenzophenone (> 98.0%), and benzhydrol (> 98.0%) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2-Hydroxy-4-methoxybenzophenone (> 98.0%), 2,2′-dihydroxy-4-methoxybenzophenone (> 98.0%) and 2-hydroxy-4-octyloxybenzophenone (> 98.0%) were purchased from Aldrich Chem. Co. (Milwaulkee, WI, U.S.A.). All other chemicals were reagent grade, obtained from commercial sources, and used without further purification.

Activation by an S-9 Fraction —— S-9 extracts (rat liver 9000 × *g* supernatant fraction induced with phenobarbital and 5,6-benzoflavone) and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). To a tube containing 990 μl of the S-9 mix (S-9 mix: 20 μl S-9, 0.8 μmol NADPH, 0.8 μmol NADH, 1.0 μmol glucose-6-phosphate, 0.4 U G6PDH, 20 μmol Na_2HPO_4, 20 μmol NaH_2PO_4, 6.6 μmol KCl and 1.6 μmol $MgCl_2$) 10 μl of each test chemical dissolved in dimethyl sulfoxide (DMSO) was added and then incubated at 37°C for 4 hr. The chemicals, after incubation with the S-9 mix, were stored at −80°C until their application to the yeast two-hybrid strain. The heat-inactivated S-9 extract was prepared by incubation at 95°C for 5 min, and used for the negative control experiments. The structures of chemicals examined in this paper are shown in Fig. 1.

Yeast Two-Hybrid Assay for Detecting Estrogenic Activity after Metabolic Activation —— In this study, we used the yeast two-hybrid system with the estrogen receptor, estrogen recepter α (ERα), and the coactivator, transcriptional intermediary factor 2 (TIF2), as previously described.[8,9] Yeast cells carrying the pGBT9-estrogen receptor ligand binding domain (pGBT9-ERLBD) and pGAD424-TIF2 plasmids were grown overnight at 30°C with vigorous shaking in selective medium (S.D. medium lacking tryptophan and leucine). The yeast cells, resuspended in 2 × S.D. medium made up at twice the usual concentration, were mixed at a 1 : 1 (v/v) ratio with the test chemicals, which had been treated with the S-9 mix, and then incubated at 30°C for 4 hr. Aliquots of cells were withdrawn and washed by centrifugation. The cell density was determined by measurement of the absorbance at 595 nm. A lysate was prepared by enzymatic digestion of the cells with 1 mg/ml Zymolyase 20T at 37°C for 15 min. The lysate (200 μl) was mixed with 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (40 μl) and incubated at 30°C for 30 min. The enzymatic reaction was stopped by the addition of 1 M Na_2CO_3 (100 μl). β-Galactosidase activity was calculated as described previously.[8] Estrogenic activity was also tested by MCF-7 proliferation assay[18] and a reporter gene assay using HeLa cells.[19]

HPLC Analysis of BZ-3 Metabolites —— BZ-3 (1.0×10^{-4} M) was incubated with the S-9 mix as mentioned above. The metabolites after S-9 activation (1.5 ml) were extracted twice with 3.0 ml ethylacetate. The extracts were dried under an N_2 stream and dissolved in 30 μl methanol. The extracts (15 μl) were applied to a reverse phase HPLC column (Cadenza CD-C18, 4.6 × 250 mm, 3 μm; Imtakt, Kyoto, Japan). LC-10AD pumps were used with a DGU-14A degassing unit and C-R7A integrator (Shimadzu, Kyoto, Japan). The HPLC column was eluted with a 75% methanol/water at a flow rate of

1.0 ml/min. The eluate was monitored at 230 nm with an SPD-10AV detector (Shimadzu). Each 0.5 ml fraction was collected and dried under an N_2 stream, and dissolved in 10 μl DMSO to be applied to the yeast two-hybrid strain for testing estrogenic activity.

Liquid Chromatography/Mass Spectrometry Analysis of BZ-3 Metabolites —— Liquid chromatography/mass spectrometry (LC/MS) analysis was performed on an API3000 (Applied Biosystems, Foster City, CA, U.S.A.) equipped with an electrospray ionization (ESI) interface and an Agilent 1100 series HPLC from Agilent Technologies (Waldbronn, Germany). The HPLC system consisted of a G1312A HPLC binary pump, a G1367A autosampler and a G1379A degasser. The column used was a reverse phase HPLC column (Cadenza CD-C18, 2.0 × 100 mm, 3 μm; Imtakt). The mobile phases consisted of 100% acetonitrile (A) and 1% aqueous acetic acid (B). Elution was performed using a linear gradient from 30% A to 80% A during 30 min at 0.2 ml/min. The ESI interface was control by Analyst software (v.1.2). ESI-MS was operated in negative or positive ion mode. The heated capillary and voltage were maintained at 500°C with and –/+4.2 kV (negative/positive mode), respectively. Mass spectra were measured from *m/z* 50 up to *m/z* 300.

RESULTS

Estrogenic Activity of Metabolites of Benzophenone Derivatives

The negative control experiments were performed using an S-9 mix containing inactive S-9 extracts (an inactive S-9 mix). Serial dilutions of E_2 were incubated with the inactive S-9 mix at 37°C for 4 hr, and then the reaction mixtures were applied to the yeast two-hybrid assay. Maximum β-galactosidase activity induced by incubation with E_2 was obtained at concentrations of 1.0×10^{-8} M and higher (Fig. 2a). The concentration of E_2 showing 10% of the 1.0×10^{-7} M activity (relative effective concentration, REC_{10}) was 1.7×10^{-10} M. Under these conditions BZ-1 and 2,3,4-trihydroxybenzophenone exhibited estrogenic activities. Their REC_{10} values were 6.5×10^{-7} and 6.8×10^{-6} M, respectively. Benzophenone, BZ-3, BZ-8, BZ-12, benzhydrol and MXC did not exhibit estrogenic activities.

The metabolic activation experiments were performed using an S-9 mix containing active S-9 extracts (an active S-9 mix). Maximum β-galactosidase activity induced by incubation with E_2 was obtained at concentrations of 1.0×10^{-8} M and higher (Fig. 2b). The REC_{10} of E_2 was 2.5×10^{-10} M. MXC, benzophenone, BZ-3 and BZ-8 exhibited estrogenic activities after incubation with the active S-9 mix. Their REC_{10} values were 2.5×10^{-5}, 1.5×10^{-4}, 1.4×10^{-5} and 1.0×10^{-5} M, respectively. The estrogenicities of BZ-1 and 2,3,4-trihydroxybenzophenone were reduced by incubation with the active S-9 mix. Their REC_{10} values were 1.5×10^{-4} and 2.1×10^{-5} M, respectively.

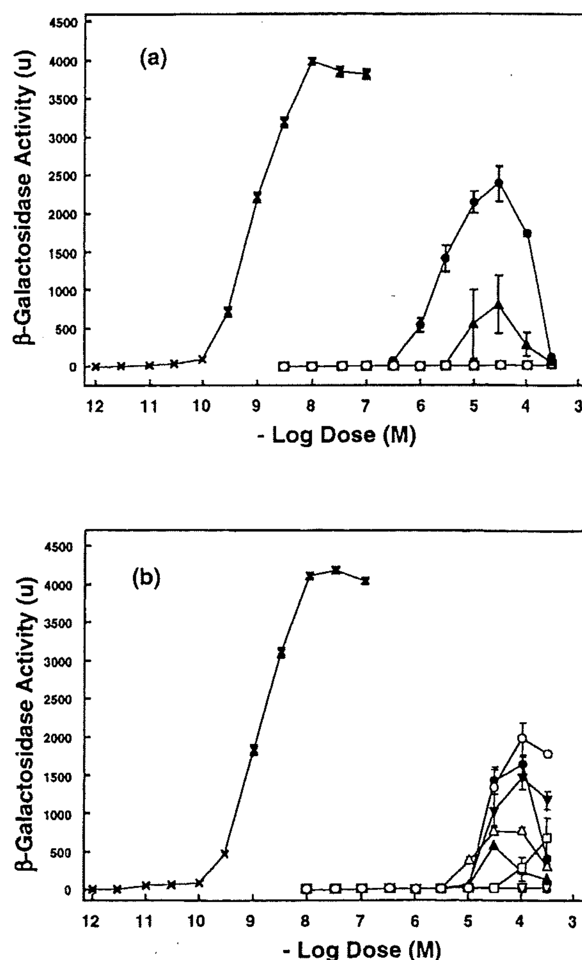

Fig. 2. Dose–Response Curve for Chemicals Incubated in Inactive S-9 Mix (a) and in Active S-9 Mix (b)

Values are means of three separate experiments (bars: S.D.). Benzophenone, (□); BZ-3, (○); BZ-8, (△); BZ-12, (▽); BZ-1,(●); 2,3,4-trihydroxybenzophenone, (▲); benzhydrol, (■); MXC, (▼); E2, (×).

HPLC Analysis of the Metabolites of BZ-3

After incubation of 1.0×10^{-4} M BZ-3 with the active S-9 mix, the metabolites were extracted with ethylacetate and then fractionated by HPLC. Three

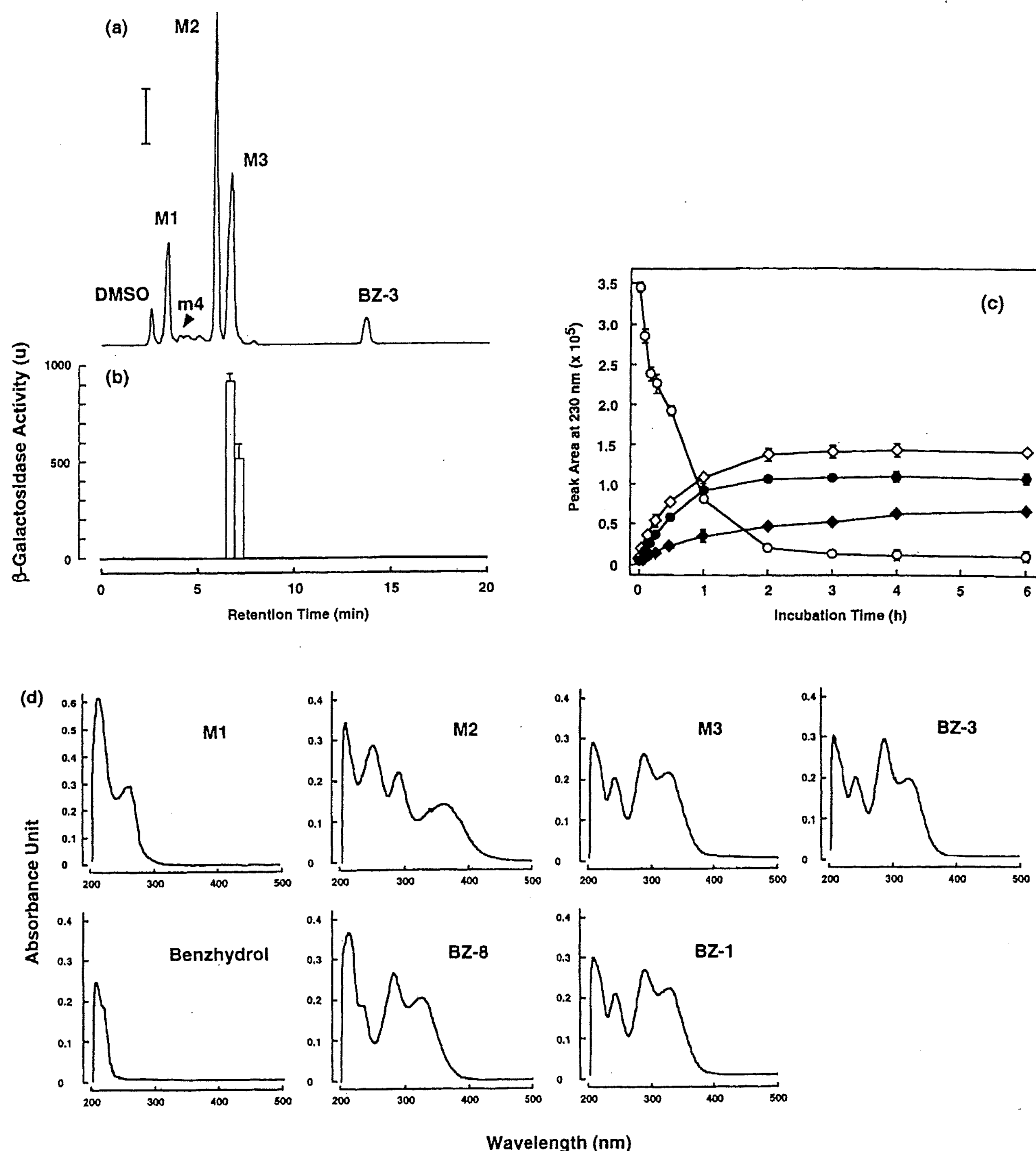

Fig. 3. The HPLC Analysis of the Metabolites of BZ-3 (a)

Bar expresses 0.2 auf. at 230 nm. The retention times were as follows (min): DMSO, 2.6; M1, 3.5; m4 (2,3,4-trihydroxybenzophenone), 4.1; M2, 5.9; M3 (BZ-1), 6.8; BZ-8, 8.1; BZ-3, 13.8. The estrogenic activities of the fractions (b). Values are means of three separate experiments (bars: S.D.). Changes in the levels of BZ-3 and its major metabolites, M1, M2 and M3 (c). Points express the peak area of recorded at 230 nm. Values are means of three separate experiments (bars: S.D.). BZ-3, (○); M1, (◆); M2, (◇); M3,(●). The concentration of BZ-3 at zero time was $9.5 \pm 0.1 \times 10^{-5}$ M. The UV spectra of M1, M2, M3, BZ-3, benzhydrol, BZ-8 and BZ-1 in methanol (d).

major metabolites (M1, M2 and M3) were detected (Fig. 3a). The retention time of a minor metabolite (m4) corresponded to that of 2,3,4-trihydroxy-benzophenone. Each fraction was also examined for its estrogenic activity using the yeast assay system. The fractions containing M3 exhibited estrogenic activities (Fig. 3b). M1, M2 and M3 produced from BZ-3 concurrently (Fig. 3c). UV spectra of metabo-

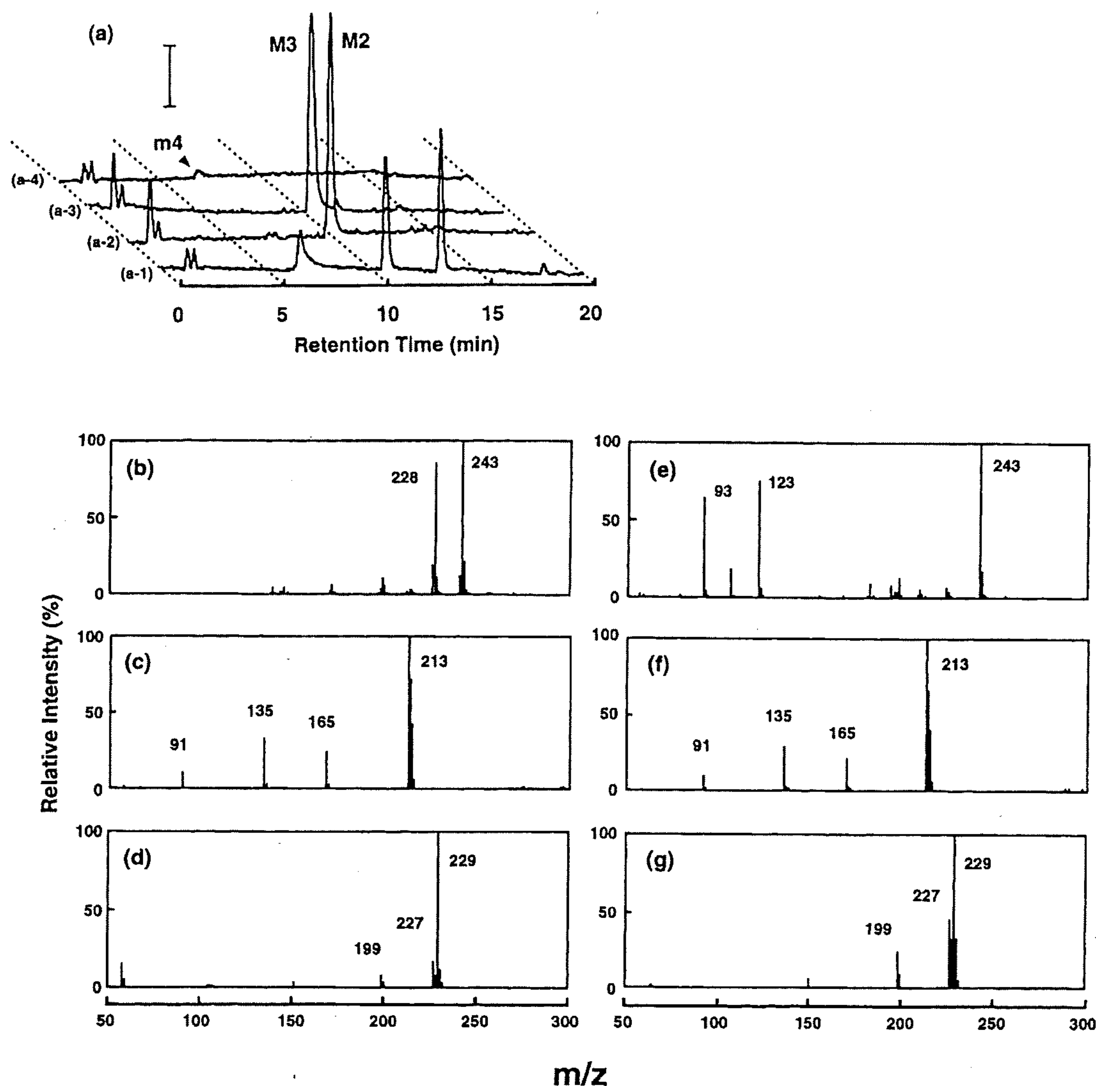

Fig. 4. Total Ion Chromatograms of BZ-3, BZ-8, BZ-1, 2,3,4-Trihydroxybenzophenone, M2, M3 and m4 (a)

Mixture of benzophenone derivatives containing 2.0×10^{-5} M BZ-3, BZ-8, BZ-1, and 2,3,4-trihydroxybenzophenone (a-1), M2 (a-2), M3 (a-3), m4 (a-4). Bar expresses the intensity of 1.0×10^{8} cps. The retention times were as follows (min): m4 (2,3,4-trihydroxybenzophenone), 6.7; M2, 10.0; M3 (BZ-1), 10.9; BZ-8, 13.5; BZ-3, 18.5. The mass spectra of M2 (b), M3 (c), m4 (d), BZ-8 (e), BZ-1 (f) and 2,3,4-trihydroxybenzophenone (g).

lites, BZ-3, benzhydrol, BZ-8 and BZ-1 in methanol were exhibited in Fig. 3d. Both benzhydrol and M1 did not have absorption above 280 nm. M2 absorbed from 200 to 440 nm with maxima at 290 and 360 nm and minima at 277 and 326 nm. M3 absorbed from 200 to 400 nm with maxima at 289 and 325 nm and minima at 262 and 312 nm. The profiles of UV spectrum and retention time in HPLC analysis of M3 were identical to those of BZ-1. After incubation of 1.0×10^{-4} M BZ-3 with the active S-9 mix for 4 hr, the concentrations of M3 (BZ-1) and BZ-3 were $3.3 \pm 0.1 \times 10^{-5}$ and $2.0 \pm 0.2 \times 10^{-6}$ M, respectively. To obtain further information about the metabolites, LC/MS analysis was also performed. The total ion chromatograms (negative ion mode) of M2, M3 and m4 were exhibited Fig. 4a. The mass spectra of M2, M3, m4, BZ-1 and 2,3,4-trihydroxybenzophenone were shown in Figs. 4b–4g, respectively. The base peak was detected at *m/z* 243 in the mass spectrum of M2. The mass spectra of M3 and m4 were identical to those of BZ-1 and 2,3,4-trihydroxybenzophenone, respectively. M1 did not detectable in both negative and positive ion mode.

DISCUSSION

P450 enzymes in the active S-9 mix are able to convert E_2 to its oxidative metabolites.[20] The dose–

response curves of E_2 after the incubation with the active S-9 mix were almost identical to that after the incubation with the inactive S-9 mix containing medium (Figs. 2a and b). Under these conditions, degradation of E_2 in the active S-9 mix containing medium was not observed (data not shown). The active S-9 mix fraction is able to mix with yeast cells suspended in 2 × S.D. medium without the need for additional measures.

The hydroxyl group of E_2 at the 3-position plays an important role in activating the ER.[21] Without the S-9 mix activation, benzophenone derivatives with a hydroxyl group at the 4-position, BZ-1 and 2,3,4-trihydroxybenzophenone, exhibited estrogenic activities. Some compounds containing phenol residues, such as *p*-alkylphenols, parabens and bisphenol A, exhibit estrogenic activities.[9,22–24] The phenol residues of these compounds are believed to participate in mimicking E_2 at the ER ligand-binding domain.[9] The phenol residue in benzophenone derivatives would also play such a role in activating ER. MXC, benzophenone, BZ-3 and BZ-8 exhibited estrogenic activities after incubation with the active S-9 mix. MXC is designated as a proestrogen for its conversion to an estrogenic metabolite, 2,2-*bis*(hydroxyphenyl)-1,1,1-trichloroethane.[12] It was also confirmed by us that benzophenone after metabolic activation showed estrogenic activity as well in the proliferation assay and the reporter gene assay using cultured cells (data not shown). This assay system is applicable for the detection of the proestrogens. We demonstrated that BZ-3 was converted to an estrogenic metabolite BZ-1 by incubation with the active S-9 mix. BZ-8 has an additional hydroxyl group at 2′-position of BZ-3. The estrogenic activities of its metabolites would depend on 2,2′,4-trihydroxybenzophenone and/or metabolites with hydroxyl group at a 4′-position. With both incubation with the inactive and the active S-9 mix, BZ-12 exhibited no estrogenic activity. The bulky octyloxy group moiety may prevent hydroxylation and/or ER activation.

BZ-3 is metabolized to BZ-1, BZ-8 and 2,3,4-trihydroxybenzophenone *in vivo*.[2–5] In the assay system, BZ-3 was metabolized to M1, M2, M3 (BZ-1) and m4 (2,3,4-trihydroxybenzophenone). The structures of M1 and M2, non-estrogenic metabolites, were inferred by examination of their UV spectra and LC/MS analysis. M1 was assumed to be a benzhydrol derivative for its lack of absorption above 280 nm. Benzhydrol could be one of the major metabolites of benzophenone in hepatocytes,[2–5] and had no absorption above 250 nm in methanol. Instead of formation of BZ-8, an unknown metabolite, M2, was detected. Compared to BZ-3, the UV spectrum of M2 was shifted to a longer wavelength. The mass spectrum suggested a molecular weight (M.W.) for M2 of 244. The M.W. of BZ-3 is 228.2. The UV and mass spectra of M2 were quite different from those of BZ-8 (Figs. 3d, 4b and 4e). M2 would be formed by the hydroxylation in the aromatic ring with methoxy and hydroxyl groups of BZ-3.

BZ-3 is used in many cosmetics and sunscreens as a UV-absorber.[1] The compound can be absorbed topically and converted to an estrogenic metabolite, BZ-1.[25,26] We demonstrated that BZ-3 was converted to the estrogenic metabolite, BZ-1, in a 33% yield by incubation with S-9 mix for 4 hr. At the same condition, 2,3,4-trihydroxybenzophenone was produced in a less than 1% yield (data not shown). Thus, the yield of non-estrogenic metabolites including M1 and M2 based on BZ-3 was approximately 60%. From 1 to 10% of BZ-3 in cosmetic products penetrates human skin.[25,26] These facts suggest that BZ-1 is produced *in vivo* by those applying a sunscreen or a cosmetic containing BZ-3. UV absorbers are increasingly used as a result of growing concern about UV irradiation and skin cancer. Schlumpf, *et al.* reported that other UV absorbers, such as 4-methyl-benzylidene camphor and octyl-methoxycinnamate, also exhibit estrogenic activity.[27] Studies of the effects on endocrine systems by UV absorbers should be performed more extensively, because of their use in children.

This assay system was able to detect the conversion of BZ-3 to an estrogenic metabolite in a minimum number of steps. This yeast two-hybrid system is able to evaluate the effects of chemicals on thyroid hormone receptors and androgen receptors by changing pairs of the receptors and coactivators to the relevant pairs.[8] Studies of the thyromimetic and anti-thyromimetic activities of metabolites of chemicals are ongoing in our laboratory. This assay system will be a useful tool for the detection of prohormonal activities of chemicals.

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Detection of Thyroid Hormone Receptor-Binding Activities of Chemicals Using a Yeast Two-Hybrid Assay

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The adverse effects of chemicals exerted *via* estrogen receptors (ER) and androgen receptors (AR) have been studied extensively in recent years. However, those occurring *via* thyroid hormone receptors (TR) have not been studied enough. We examined the TR-binding activities of thyronine derivatives and alkylphenol derivatives (bisphenol A, parabens and antioxidants with *o-t*-butylphenol residue) using a yeast two-hybrid assay. In this assay system, the TR-binding activity of 3,5,3′-triiodo-L-thyronine (T_3) was detectable at concentrations as low as 3.0×10^{-8} M and reached a plateau at 1.0×10^{-6} M. The concentration of T_3 producing 10% of the activity stimulated at 1.0×10^{-6} M (10% relative effective concentration, REC_{10}) was 2.1×10^{-8} M. 3,5,3′,5′-tetraiodo-L-thyronine (T_4), 3,3′,5′-triiodo-L-thyronine (rT_3) and 3, 5-diiodo-L-thyronine (T_2) also exhibited TR-binding activities. The REC_{10} values of these chemicals were 4.2×10^{-8}, 5.0×10^{-7} and 1.0×10^{-5} M, respectively. *o*-Isopropylphenol and *o-t*-butylphenol exhibited TR-binding activities with REC_{10} values of 3.1×10^{-4} and 4.8×10^{-5} M, respectively, whereas *t*-butylbenzene, isopropylbenzene and the other chemicals tested had no detectable TR-bindng activity. These results suggest that a phenolic hydroxyl group and the *ortho*-substituents may play important roles in the TR-binding activities of these chemicals. This assay system will be a useful tool for screening the TR-binding activities of chemicals.

Key words —— thyroid hormone, yeast two-hybrid assay, phenolic hydroxyl group, *o*-isopropylphenol, *o-t*-butylphenol

INTRODUCTION

Since the publication of "Our Stolen Future"[1] in 1996, there has been considerable concern about the disruptive effects of chemicals on the endocrine systems of humans and wild life, especially *via* the hormone receptors.[2] Chemicals with such effects are referred to as endocrine disruptors (EDs). The effects of chemicals on the function of steroid hormones, especially estrogen and androgen, have been studied extensively.[3–6] Most of the activity of steroid hormones is mediated through their receptors, which reside in the cell nucleus and regulate the transcription of target genes in a ligand-dependent manner.[7] Nuclear hormone receptors constitute a large superfamily of ligand-inducible transcriptional factors.[8] TR is a member of this superfamily as are the receptors for steroid hormones. Thyroid hormone fulfils an important role in the development of neurons, multiplication of cells, cell death and energy metabolism of many organisms.[9] Thus, there may be chemicals which perturb the endocrine system by modulating TR function. For relevant examples, it is doubted that the dioxins and polychlorinated biphenyls (PCBs) produce their adverse effects by disrupting thyroid hormone function directly.[10,11] Compared to the numerous reports regarding estrogen receptors (ER)[3–5] and androgen receptors (AR),[2,6] there are few concerning the biological effects of chemicals that act through thyroid hormone receptors (TR).

As a method for evaluating the thyromimetic activities of chemicals, a frog metamorphosis assay[12] and a thyroid hormone receptor binding assay[10] have been established. We have developed a novel screening method for chemicals with hormonal activities

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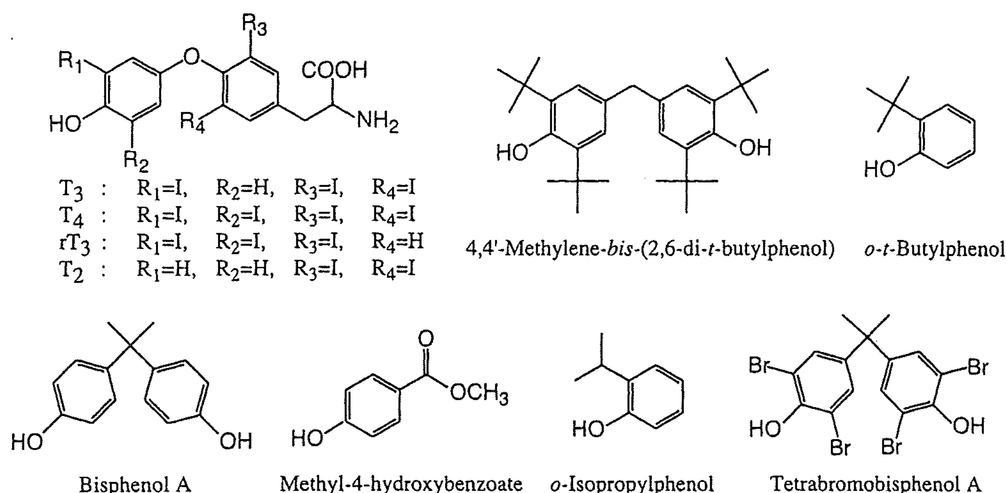

Fig. 1. Typical Structures of the Chemicals Tested in the Yeast-Two-Hybrid Assay

using a yeast two-hybrid system and demonstrated that this method detected the ER-, AR- and TR-binding activities of chemicals.[13] We tested the ER-binding activities of 517 chemicals using the yeast two-hybrid assay.[14] In that manuscript, we reported that a phenol with a hydrophobic moiety at the *para*-position is a key structural motif of ER-binding chemicals. These results were supported by the findings obtained from a crystallographic study of the ligand-binding ER. The study reported that the structural features of 17-β-estradiol (E_2), a hydrophobic steroid skeleton with a phenolic hydroxyl group, played a crucial role in the interaction between ER and E_2.[15] There is a reasonable relationship between the results obtained from the yeast two-hybrid assay and findings from the crystallographic study. Wagner *et al.* performed crystallographic analysis of TR and its ligands and reported that a phenol group in the ligands also played a key role in activation of the receptor.[16] For designing an iodo-free drug with thyromimetic activity, an *o*-isopropylphenol residue is useful for optimization of the activity.[17] Hydroxylated PCBs compete with 3,5,3′,5′-tetraiodo-L-thyronine (T_4) for the binding to transthyretin to perturb the thyronine hormone homeostasis.[18] Thus, there is relevance to studies of the TR-binding activities of chemicals with a phenol group. There are few reports concerning the thyromimetic activities of chemicals. Here, we examined the TR-binding activities of chemicals with a phenol group, such as alkylphenols, bisphenol A, tetrabromobisphenol A and antioxidants with a *t*-butylphenol residue, as well as thyronines, by our yeast two-hybrid assay.

MATERIALS AND METHODS

Chemicals —— 3,5,3′-Triiodo-L-thyronine (T_3; > 97%) was purchased from Fluca (Steinheim, German). T_4 was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, U.S.A.). 3,3′,5′-Triiodo-L-thyronine (rT_3) and 3,5-diiodo-L-thyronine (T_2) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-Thyronine was purchased from Bachem AG (Bubendorf, Switzerland). All other chemicals were reagent grade and used without further purification. The chemical structures tested in the yeast two-hybrid assay are shown in Fig. 1.

The Yeast Two-Hybrid Assay —— The TR-binding activities of chemicals were examined with a yeast two-hybrid assay system with the thyroid hormone receptor, TRα, and the coactivator, transcriptional intermediary factor 2 (TIF2), as described previously.[13] Minor modifications of the duration of test chemical incubation were made in order to raise the assay sensitivity. The yeast expression plasmids, pGBT9 and pGAD424, were purchased from Clontech (Palo Alto, CA, U.S.A.). The LBD of TRα (codons 173-461) was amplified by reverse transcription (RT)-PCR using total RNA from rat liver. The *Eco*RI and *Bam*HI sites were introduced in 5′ and 3′ terminus of amplified fragments and subcloned into *Eco*RI-*Bam*HI sites of pGBT9 so that they were in the same translational reading frame as the vector's GAL4 DNA binding domain (GAL4DBD). The receptor interaction domain (RID) of coactivator, TIF2, was amplified by PCR from cDNAs[19] and subcloned into *Eco*RI-*Bam*HI-*digested* pGAD424 for the production of fusion pro-

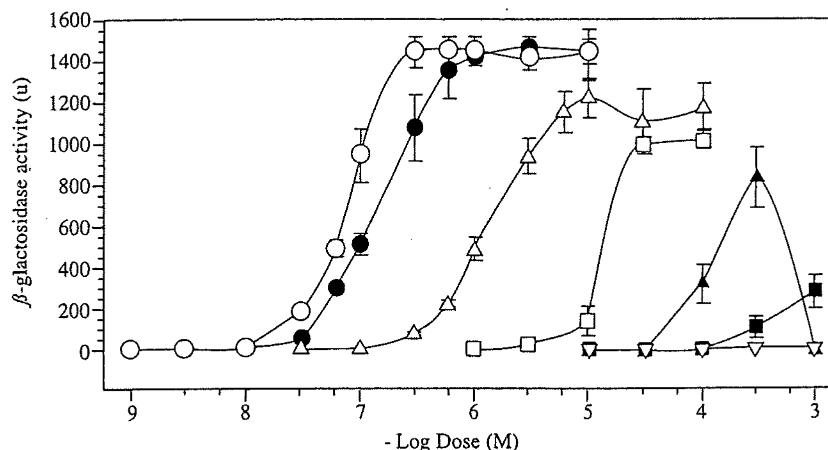

Fig. 2. Dose–Response Curves for the TR-Binding Activities of Thyronine Derivatives, *o*-Isopropylphenol and *o*-*t*-Butylphenol

The points represent averages β-galactosidase activities obtained from three independent experiments on separate days. Bars represent S.D. T_3 (○); T_4 (●); rT_3 (△); T_2 (□); *o*-*t*-butylphenol (▲); *o*-isopropylphenol (■) ; thyronine (▽).

teins with the GAL4 activation domain (GAL4AD). All sequences generated by PCR were confirmed by DNA sequencing. The yeast strain used in this study was Y190 (*MATa, ura3-52, his-D200, ade2-101, trp1-901, leu2-3, 112, gal4Dgal80D, URA3:::GAL-lacZ, cyhr2, LYS2:::GAL-HIS3*), obtaind from Clontech (Palo Alto). Yeast cells were transformed with the pGBT9-TRα and pGAD424-TIF2 using lithium acetate method and selected by growth on synthetic dropout (SD) medium (lacking tryptophan and leucine). Yeast transformants were preincubated overnight at 30°C in SD medium free from tryptophan and leucine. The culture (250 μl) was then mixed in a small test tube with a DMSO solution (2.5 μl) of test chemical and incubated for 24 hr at 30°C. After washing by centrifugation, the cells were digested enzymatically by incubation with 1 mg/ml Zymolyase 20T (100 μl) at 37°C for 15 min. The lysate was mixed with 4 mg/ml *o*-nitrophenyl-β-D-galactoside (40 μl) and reacted at 30°C until development of the yellow color before the reaction was stopped by the addition of 1 M Na_2CO_3 (100 μl). β-Galactosidase activity is calculated as described previously and represents the mean ± S.D. of 3 independent experiments performed on separate days. The results were evaluated by relative activity, expressed as 10% relative effective concentration (REC_{10}), that is the concentration of the test chemical showing 10% of the agonist activity of 1.0×10^{-6} M T_3, which is the optimum concentration for T_3. When the activity of test chemical was higher than REC_{10} within the concentration tested, we judged the chemical as positive. When it was judged to be negative, the highest dose tested is indicated.

RESULTS

The β-galactosidase activities induced by T_3 were detectable from 3.0×10^{-8} M and reached a plateau at 1.0×10^{-6} M. The β-galactosidase activity obtained by incubation with 1.0×10^{-6} M T_3 was 1460 ± 60 u (Fig. 2). REC_{10} value of T_3 was 2.1×10^{-8} M. Except for thyronine, thyronine derivatives T_4, rT_3 and T_2, also exhibited TR-binding activities. Their REC_{10} values are listed in Table 1. The maximal activity induced by T_4 reached that of T_3 at 1.0×10^{-6} M and higher. The activity induced by rT_3 and T_2 reached a plateau from 10^{-5} M and 3.0×10^{-5} M at around 80 and 70% of 1.0×10^{-6} M T_3, respectively. Tyrosine and its iodinated derivatives did not exhibit TR-binding activities within their soluble concentration. However, 3.0×10^{-4} M 3,5-dibromotyrosine exhibited weak TR-binding activity (less than 10% activity of 1.0×10^{-6} M T_3).

Among alkylphenols, *p*-hydroxybenzoates and bisphenol A derivatives were examined, only *o*-isopropylphenol and *o*-*t*-butylphenol exhibited TR-binding activities (Fig. 2). The REC_{10} values of *o*-isopropylphenol and *o*-*t*-butylphenol were 3.1×10^{-4} and 4.8×10^{-5} M, respectively. Cytotoxic effects of these chemicals were observed at 1.0×10^{-3} M and higher. Other alkylphenols, *p*-hydroxybenzoates and bisphenol A derivatives did not exhibit TR-binding activities. Isopropylbenzene and *t*-butylbenzene also did not exhibit TR-binding activities. Chemicals with *t*-butylphenol residue, which are used as antioxidants commercially were examined and found to be negative in this assay system (Table 1).

Table 1. Detection of TR-Binding Activities of Chemicals Using a Yeast Two-Hybrid Assay

	Compounds	$REC_{10}(M)^{a)}$
Thyronine derivatives	3,5,3′-Triiodo-L-thyronine (T_3)	2.1×10^{-8}
	Thyroxine (T_4)	4.2×10^{-8}
	3,3′,5′-Triiodo-L-thyronine (rT_3)	5.0×10^{-7}
	3,5-Diiodo-L-thyronine (T_2)	1.0×10^{-5}
	L-Thyronine	$> 1.0 \times 10^{-4}$
Thyrosine derivatives	Thyrosine	$> 3.0 \times 10^{-4}$
	3-Iodotyrosine	$> 1.0 \times 10^{-5}$
	3,5-Diiodo-tyrosine	$> 1.0 \times 10^{-4}$
	3,5-Dibromo-tyrosine	$> 3.0 \times 10^{-4}$ b)
Phenols	*p*-Methylphenol	$> 3.0 \times 10^{-4}$
	p-Ethylphenol	$> 3.0 \times 10^{-4}$
	p-*n*-Propylphenol	$> 3.0 \times 10^{-4}$
	p-*s*-Butylphenol	$> 3.0 \times 10^{-4}$
	p-*t*-Butylphenol	$> 3.0 \times 10^{-4}$
	p-*n*-Butylphenol	$> 3.0 \times 10^{-4}$
	p-*t*-Pentylphenol	$> 3.0 \times 10^{-4}$
	p-*n*-Pentylphenol	$> 3.0 \times 10^{-4}$
	p-*n*-Hexylphenol	$> 3.0 \times 10^{-4}$
	p-*n*-Heptylphenol	$> 3.0 \times 10^{-4}$
	p-*t*-Octylphenol	$> 3.0 \times 10^{-4}$
	p-*n*-Octylphenol	$> 3.0 \times 10^{-4}$
	p-*br*-Nonylenol	$> 3.0 \times 10^{-4}$
	p-*n*-Dodecylphenol	$> 3.0 \times 10^{-4}$
	o-Methylphenol	$> 3.0 \times 10^{-4}$
	o-Ethylphenol	$> 3.0 \times 10^{-4}$
	o-Isopropylphenol	3.1×10^{-4}
	o-*n*-Propylphenol	$> 3.0 \times 10^{-4}$
	o-*t*-Butylphenol	4.8×10^{-5}
	o-*n*-Butylphenol	$> 3.0 \times 10^{-4}$
	o-Phenylphenol	$> 3.0 \times 10^{-4}$
	o-Iodophenol	$> 3.0 \times 10^{-4}$
	m-*t*-Butylphenol	$> 3.0 \times 10^{-4}$
Parabens	Methyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
	Ethyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
	Propyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
	Butyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
	Benzyl 4-hydroxybenzoate	$> 3.0 \times 10^{-4}$
Bisphenol A derivatives	Bisphenol A	$> 3.0 \times 10^{-4}$
	Tetrabromobisphenol A	$> 3.0 \times 10^{-4}$
Benzenes	Isopropylbenzene	$> 3.0 \times 10^{-4}$
	t-Butylbenzene	$> 3.0 \times 10^{-4}$

a) The concentration showing 10% activity of 10^{-6} M T_3 (relative activity).
b) The activity of 1.0×10^{-3} M L-Thyronine was less than 10% the activity of the 1.0×10^{-6} M T_3.

DISCUSSION

T_3 and T_4 have agonist activity at TR, whereas rT_3 and T_2 do not.[20] In this study, T_3, T_4, rT_3 and T_2 exhibited TR activating ability with the rank order of potency $T_3 > T_4 > rT_3 > T_2$. rT_3 and T_2 were partial-agonists at TR in this assay system. The substantial lengths of the TR and TIF2 were fused with

Table 1. Continued

	Compounds	REC_{10}(M)[a]
Antioxidants	BHT	$> 3.0 \times 10^{-4}$
	BHA	$> 3.0 \times 10^{-4}$
	4-Hydroxymethyl-2,6-di-*t*-butylphenol	$> 3.0 \times 10^{-4}$
	3-(3′,5′-Di-*t*-butyl-4′-hydroxyphenyl)propionic acid	$> 3.0 \times 10^{-4}$
	4-4′-Thiobis(3-methyl-6-*t*-butylphenol)	$> 3.0 \times 10^{-4}$
	4,4′-Methylenebis(2,6-di-*t*-butylphenol)	$> 3.0 \times 10^{-4}$
	2,2′-Methylenebis(4-methy-6-*t*-butylphenol)	$> 3.0 \times 10^{-4}$
	2,2′-Methylenbis(4-ethyl-6-*t*-butylphenol)	$> 3.0 \times 10^{-4}$
	1,1,3-Tris(2-methyl-4-hydroxy-5-*t*-butylphenyl)butane	$> 3.0 \times 10^{-4}$
	1,3,5-Trimethyl-2,4,6-tris(3,5-di-*t*-butyl-4-hydroxybenzyl)benzene	$> 3.0 \times 10^{-4}$
	Pentaerythritol tetrakis[3-(3′5′-di-*t*-butyl -4′-hydroxyphenyl)propionate]	$> 3.0 \times 10^{-4}$

the GAL4 DNA binding domain and activation domain, respectively. The intact TR and TIF2 were not produced in the yeast cells. This background in the assay system could be one reason for our observations.

Drug design studies on iodine-free TR agonists produced the following information: 1) a phenol group is essential for activity; 2) the amino acid residue of T_3 is not necessary; and, 3) 3′-iodine can be replaced by an isopropyl group.[17] By the crystallographic analysis of TR, it was also found that a phenol group in ligands plays an important role in activation of the receptor.[16] Thus, we conducted an examination of the TR-binding activities of alkylphenols, parabens and bisphenol A derivatives. *o*-Isopropylphenol and *o*-*t*-butylphenol exhibited activities. When β-galactosidase was induced by the interaction of these chemicals and the endogenous factor of the yeast, it was assumed that the activities did not occur *via* TR-TIF2. However, we have confirmed that these chemicals did not exhibit ER-binding activities in the yeast two-hybrid assay with ER-TIF2 (data not shown). Therefore these chemicals actually exert these actions *via* TR-TIF2. All other alkylphenols examined were negative. The size and position of alkyl group could be a restrictive factor. [4-(4-Hydroxy-3-isopropyl-benzyl)-3,5-dimethyl-phenoxy]-acetic acid,[17] 3,5-dimethyl-3′-isopropylthyronine[16] and 3,5-dibromo-3′-isopropylthyronine[16] were synthesized as iodine-free TR agonists. These chemicals adopted the isopropyl group as a substitute for iodine. In *o*-isopropylphenol and *o*-*t*-butylphenol, the isopropyl group and the *t*-butyl group seemed to mimic the iodine of the 3′ position of T_3, respectively. Interestingly, *o*-iodophenol did not exhibit activity. Iodine is an electron-accepting substituent, whereas the isopropyl and *t*-butyl groups are electron-donating substituents. The degree of dissociation of phenol may also affect the TR-binding activities of *o*-alkylphenols.

The phenolic system antioxidants, listed in Table 1, have an *o*-*t*-butylphenol residue in the molecule. These antioxidants are important industrial materials, which are widely used in foods and resins. We also evaluated the TR-binding activities of these antioxidants. No antioxidants exhibited TR-binding activities. Except for BHA and BHT, the structures of antioxidants were larger than those of thyroid hormones and *o*-*t*-butylphenol. Steric hindrance may prevent the antioxidants from binding to TR and/or activating it. These antioxidants are decomposed to the smaller molecules with *o*-*t*-butylphenol *in vivo*.[21,22] Their TR-binding activities could be examined subsequent to this report. Both butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), derivatives of 2,6-di-*t*-butylphenol, were negative. The additional substitution at the *ortho*-position of *o*-*t*-butylphenol may reduce the TR-binding activity as 5′-iodine of T_4 reduced the activity of T_3.

3,5-Dibromo-tyrosine exhibited weak TR-binding activity. This result suggests that chemicals with *o*-bromophenol residue may affect the activation of TR. Hydroxylated polybrominated biphenyls (PBBs) and tetrabromobisphenol A (TBBPA) compete with T_4 for binding to transthyretin.[23] The study of TR-binding and anti-TR-binding activities of PBBs and polybrominated dipheyl ethers (PBDEs) and their metabolites are ongoing in our laboratory.

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Estrogenic and thyroid hormone activity of a series of hydroxy-polychlorinated biphenyls

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Abstract

A series of novel synthetic monohydroxy polychlorinated biphenyls (OH-PCBs) (5 trichloro-, 5 tetrachloro- and 5 pentachloro-compounds) have been characterized (^{1}H and ^{13}C NMR and high resolution MS) and their estrogenic and thyroid hormone activities assessed using a yeast two-hybrid assay, both with and without possible metabolic activation by rat liver S9 preparation. Moderate estrogenic activity was found for 2,3,4′-trichlorobiphenyl-4-ol (compound 5) but this was eliminated when exposed to the S9 mix. 2,2′,3′,4,6-Pentachlorobiphenyl-3-ol (13) and 2′,3,3′,6-tetrachlorobiphenyl-4-ol (10) both showed weak estrogenicity in the absence of the S9 mix. The estrogenicity of compound (10) was enhanced 10-fold by exposure to S9 metabolic activation but that of compound (13) remained unchanged. 2′,4,5′,6-Tetrachlorobiphenyl-2-ol (6) showed strong thyroid hormonal activity (5% of that of T4) whereas 3′,4,6-trichlorobiphenyl-3-ol (4), compound (10) and 2,3′,4,5′,6-pentachlorobiphenyl-3-ol (14) showed moderate activity, and 2′,3,3′,5-tetrachlorobiphenyl-2-ol (8) and 3,3′,5,5′,6-pentachlorobiphenyl-2-ol (11) showed weak activity. The activity of (4) was eliminated by S9 metabolic activation whereas those of (6) and (14) were weakened and that of (10) remained unchanged.

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Keywords: Hydroxy-PCBs; Estrogenic activity; Thyroid hormonal activity; Yeast two-hybrid assay

1. Introduction

Polychlorinated biphenyls (PCBs) are industrial chemicals that have been used in electrical capacitors and transformers, as lubricants, cooling fluids, flame-retardants, and in hydraulic fluids, adhesives, plasticizers etc. They are metabolized in vivo to hydroxyl and sulfur compounds (Letcher et al., 2000) and hydroxylated metabolites have been found in human serum (Bergman et al., 1994), whole blood (Sandau et al., 2000) and plasma (Hovander et al., 2002). Hydroxylated PCBs have been shown to inhibit mitochondrial oxidative phosphorylation (Narasimhan et al., 1991), thyroid hormone sulfation (Schuur et al., 1998a,b,c), estrogen sulfotranferase (Kester et al., 2000) and the sulfation and glucuronidation of 3-hydroxy-benzo[a]pyrene (van den Hurk et al., 2002), to affect thyroxine (T4) levels (Sinjari and Darnerud, 1998) and to exhibit estrogenic or antiestrogenic activity (Korach et al., 1988; Bergeron et al., 1994; Fielden et al., 1997; Moore et al., 1997; Vakharia and Gierthy, 2000; Schultz, 2002).

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In the study reported here we have characterized 15 novel synthetic monohydroxy PCBs and assessed their estrogenic and thyroid hormone activity using a yeast two-hybrid assay (Shiraishi et al., 2000). Five trichloro-, five tetrachloro- and five pentachloro-compounds were chosen with a range of features that might provide structural information on the requirements of the estrogen and thyroid hormone receptors, and give some insight into the toxicological implications of PCB metabolism. The yeast two-hybrid assay provided a simple, quick, highly sensitive, and readily reproducible method of assaying estrogenic and thyroid hormone activity.

2. Materials and methods

2.1. Preparation and characterization of compounds 1–15

The novel monohydroxy PCBs 1–15 were synthesized by thermal diazo-coupling between a chlorophenol and a chloro-aniline diazonium salt (Okumura and Shibata, 1975) and were characterized by ^{1}H and ^{13}C NMR spectroscopy and by high resolution mass spectrometry. NMR spectra (5 mg/0.5 ml $CDCl_3$,) were recorded at 800 MHz (^{1}H) and 200 MHz (^{13}C) on a JEOL ECA-800 spectrometer (JEOL, Tokyo, Japan). Assignments of protons were made after a consideration of *ortho*- and *meta*-spin coupling and were aided in one case (compound 3) by a 2D (COSY) experiment. *Ortho*- and *meta*-spin coupling constants were measured but no attempt was made to observe and record *para*-coupling. Proton assignments were confirmed by comparing spectra with simulated spectra generated by inserting measured chemical shift and spin coupling constant data into the WINDNMR-Pro freeware program downloaded from http://www.chem.wisc.edu/areas/reich/pH/windnmr.htm. Carbon assignments were aided by 2D (HMQC) spectra and a consideration of data presented by SDBS [SDBSWeb: http://www.aist.go.jp/RIODB/SDBS (August 2002)].

High resolution mass spectra were measured by GC–MS using an HP6890A GC (Agilent Technologies, Tokyo, Japan) fitted with a HP-1MS column (Agilent) (30 m × 0.25 mm i.d. × 0.25 μm). Injector temperature was 260 °C; transfer line temperature, 260 °C; column temperature program was 60 °C (1 min) → 20 °C/min → 200 °C → 5 °C/min → 260 °C. The carrier gas was helium at 1.2 ml/min. Mass spectra were taken on a JMS-700 instrument (JEOL, Tokyo, Japan) using EI mode, an ionization current of 300 μA, ionization energy of 70 eV, and accelerator voltage of 8.0 kV.

2.2. Yeast two-hybrid assays

The agonist activities of compounds 1–15 were measured, both with and without possible metabolic activation by rat liver S9 preparation (Kikkoman Company, Noda, Japan), with (i) a yeast two-hybrid estrogenicity assay system using yeast cells (*Saccharomyces cervisiae* Y190) into which the human estrogen receptor ERa and the coactivator TIF2 had been introduced, and (ii) a yeast two-hybrid thyroid hormone activity assay using *S. cervisiae* Y190 cells into which the human thyroid hormone receptor TRa and the coactivator TIF2 had been introduced. Both were adapted to a chemiluminescent reporter gene (for β-galactosidase) method employing a 96-well culture plate (Shiraishi et al., 2000). Aliquots of test chemical solutions (1 mM in DMSO, 20 μl), after or without a period of incubation with rat liver S9 mix (37 °C, 1 h), were incubated (30 °C, 4 h) with yeast cells that had been preincubated (30 °C, overnight) in modified SD medium (lacking tryptophan and leucine). A mixed solution for inducing chemiluminesence and for enzymatic digestion (Zymolase 20T) was added followed by a light emission accelerator solution. The chemiluminesence produced by released β-galactosidase was measured with a 96-well plate luminometer (Luminescencer-JNR AB-2100, ATTO, Tokyo, Japan). Agonist activity was recorded as the $EC \times 10$ which was defined as the concentration of test solution producing a chemiluminescent signal 10× that of the blank control.

3. Results and discussion

3.1. NMR data

^{1}H chemical shifts for compounds 1–15 are given in Table 1 and *ortho*- and *meta*-spin coupling constants are given in Table 2. ^{13}C chemical shifts for compounds 1–15 are given in Table 3. ^{1}H spectra simulated using the WINDNMR-Pro program with the measured chemical shifts and measured coupling constants confirmed the structural assignments.

3.2. High resolution MS data

Measured and theoretical values for the three most abundant combinations of ^{35}Cl and ^{37}Cl for tri- tetra- and penta-chlorinated compounds are given in Table 4. In all cases the mass spectral data were consistent with the proposed molecular formulas.

3.3. Estrogenicity and thyroid hormonal activity assays

Results of yeast two-hybrid assays of compounds 1–15 for estrogenicity and thyroid hormone activity are presented in Table 5 and Figs. 1 (estrogenicity) and 2 (thyroid activity). Moderate estrogenic activity was found for 2,3′,4′-trichlorobiphenyl-4-ol (compound 5)